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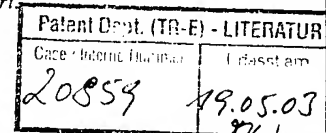
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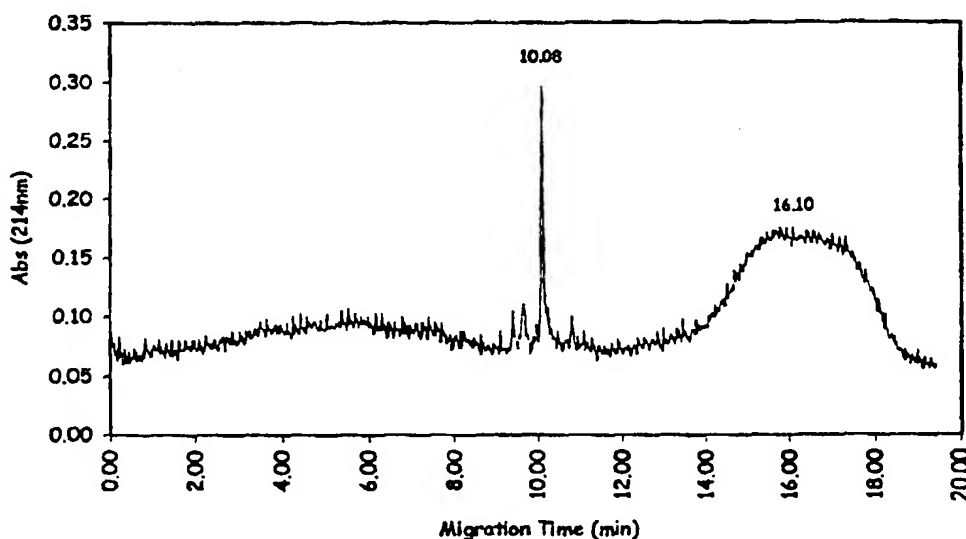
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(71) Applicant (for all designated States except US): APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. [NL/NL]; 14 John B. Gorsiraweg, Curacao (AN).		
(72) Inventors; and (75) Inventors/Applicants (for US only): EL TAYAR, Nabil [CH/US]; 143 Gerald Road, Milton, MA 02186 (US). ROBERTS, Michael, J. [US/US]; 104 Cane Brook Court, Madison, AL 35758 (US). HARRIS, Milton [US/US]; 3119 Highland Plaza, Huntsville, AL 35801 (US). SAWLIVICH, Wayne [US/US]; 24 Morse Avenue, Wilmington, MA 01887 (US).		
(74) Agent: YUN, Allen, C.; Browdy and Neimark, P.L.L.C., Suite 300, 419 Seventh Street, N.W., Washington, DC 20004 (US).		Published Without international search report and to be republished upon receipt of that report.



(54) Title: POLYOL-IFN-BETA CONJUGATES



(57) Abstract

PEG-IFN- β conjugates, where a PEG moiety is covalently bound to Cys¹⁷ of human IFN- β , are produced by a process of site specific PEGylation with a thiol reactive PEGylating agent. A pharmaceutical composition and a method for treating infections, tumors and autoimmune and inflammatory diseases are also provided. The invention further relates to a method for the stepwise attachment of PEG moieties in series to a polypeptide, and more particularly to IFN- β .

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POLYOL-IFN-BETA CONJUGATES

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority under 35 U.S.C. §119(e) from U.S. provisional application no. 60/083,339, 5 the entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates to polyol-IFN- β conjugates wherein a polyol unit is covalently bound to Cys¹⁷. Further 10 objects of the present invention are the process for their site-specific production as well as their use in the therapy, prognosis or diagnosis of bacterial infections, viral infections, autoimmune diseases and inflammatory diseases. The present invention further relates to a method for the stepwise 15 attachment of two or more PEG moieties to a polypeptide.

BACKGROUND OF THE INVENTION

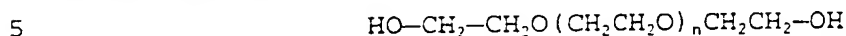
Human fibroblast interferon (IFN- β) has antiviral activity and can also stimulate natural killer cells against neoplastic cells. It is a polypeptide of about 20,000 Da 20 induced by viruses and double-stranded RNAs. From the nucleotide sequence of the gene for fibroblast interferon, cloned by recombinant DNA technology, Derynk et al. (Nature, 285:542-547, 1980) deduced the complete amino acid sequence of the protein. It is 166 amino acid long.

25 Shepard et al. (Nature, 294:563-565, 1981) described a mutation at base 842 (Cys \rightarrow Tyr at position 141) that abolished its anti-viral activity, and a variant clone with a deletion of nucleotides 1119-1121.

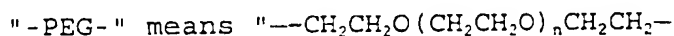
Mark et al. (Proc. Natl. Acad. Sci. U.S.A., 30 81(18):5662-5666, 1984) inserted an artificial mutation by replacing base 469 (T) with (A) causing an amino acid switch from Cys \rightarrow Ser at position 17. The resulting IFN- β was reported to be as active as the 'native' IFN- β and stable during long-term storage (-70°C).

35 Covalent attachment of the hydrophilic polymer

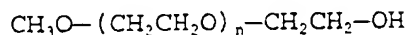
polyethylene glycol, (PEG), also known as polyethylene oxide, (PEO), to molecules has important applications in biotechnology and medicine. In its most common form, PEG is a linear polymer having hydroxyl groups at each terminus:



This formula can be represented in brief as HO-PEG-OH, where it is meant that -PEG- represents the polymer backbone without the terminal groups:



10 PEG is commonly used as methoxy-PEG-OH, (m-PEG), in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group that is subject to chemical modification.



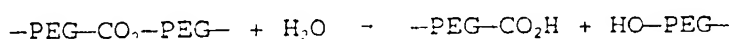
15 Branched PEGs are also in common use. The branched PEGs can be represented as $\text{R}(-\text{PEG}-\text{OH})_m$ in which R represents a central core moiety such as pentaerythritol or glycerol, and m represents the number of branching arms. The number of branching arms (m) can range from three to a hundred or more.

20 The hydroxyl groups are subject to chemical modification.

Another branched form, such as that described in PCT patent application WO 96/21469, has a single terminus that is subject to chemical modification. This type of PEG can be represented as $(\text{CH}_3\text{O}-\text{PEG}-)_p\text{R}-\text{X}$, whereby p equals 2 or 3, R represents a central core such as lysine or glycerol, and X represents a functional group such as carboxyl that is subject to chemical activation. Yet another branched form, the "pendant PEG", has reactive groups, such as carboxyl, along the PEG backbone rather than at the end of PEG chains.

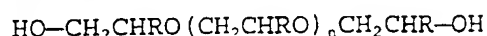
30 In addition to these forms of PEG, the polymer can also be prepared with weak or degradable linkages in the backbone. For example, Harris has shown in U.S. Patent Application 06/026,716 that PEG can be prepared with ester linkages in the polymer backbone that are subject to hydrolysis.

35 This hydrolysis results in cleavage of the polymer into fragments of lower molecular weight, according to the reaction scheme:



According to the present invention, the term polyethylene glycol or PEG is meant to comprise all the above described derivatives.

- 5 The copolymers of ethylene oxide and propylene oxide are closely related to PEG in their chemistry, and they can be used instead of PEG in many of its applications. They have the following general formula:



- 10 wherein R is H or CH₃.

PEG is a useful polymer having the property of high water solubility as well as high solubility in many organic solvents. PEG is also non-toxic and non-immunogenic. When PEG is chemically attached (PEGylation) to a water insoluble
15 compound, the resulting conjugate generally is water soluble, as well as soluble in many organic solvents.

PEG-protein conjugates are currently being used in protein replacement therapies and for other therapeutic uses. For example, PEGylated adenosine deaminase (ADAGEN®) is being
20 used to treat severe combined immunodeficiency disease (SCIDS), PEGylated L-asparaginase (ONCAPSPAR®) is being used to treat acute lymphoblastic leukemia (ALL), and PEGylated interferon-α (INTRON(R) A) is in Phase III trials for treating hepatitis C.

For a general review of PEG-protein conjugates with
25 clinical efficacy see N.L. Burnham, Am. J. Hosp. Pharm., 15:210-218, 1994.

A variety of methods have been developed to PEGylate proteins. Attaching PEG to reactive groups found on the protein is typically done utilizing electrophilically activated PEG
30 derivatives. Attaching PEG to the α- and ε-amino groups found on lysine residues and the N-terminus results in a conjugate consisting of a mixture of products.

Generally, such conjugates consist of a population of the several PEG molecules attached per protein molecule
35 ("PEGmers") ranging from zero to the number of amino groups in the protein. For a protein molecule that has been singly modified, the PEG unit may be attached at a number of different amine sites.

This type of non-specific PEGylation has resulted in a number of conjugates that become almost inactive. Reduction of activity is typically caused by shielding the protein's active binding domain as is the case with many cytokines and antibodies. For example, Katre et al. in U.S. Patent 4,766,106 and U.S. Patent 4,917,888 describe the PEGylation of IFN- β and IL-2 with a large excess of methoxy-polyethylene glycolyl N-succinimidyl glutarate and methoxy-polyethylene glycolyl N-succinimidyl succinate. Both proteins were produced in microbial host cells, which allowed the site-specific mutation of the free cysteine to a serine. The mutation was necessary in microbial expression of IFN- β to facilitate protein folding. In particular, the IFN- β used in these experiments is the commercial product Betaseron[®], in which Cys¹⁷ residue is replaced with a serine. Additionally, the absence of glycosylation reduced its solubility in aqueous solution. Non-specific PEGylation resulted in increased solubility, but a major problem was the reduced level of activity and yield.

European Patent Application EP 593 868, entitled PEG-Interferon Conjugates, describes the preparation of PEG-IFN- α conjugates. However, the PEGylation reaction is not site-specific, and therefore a mixture of positional isomers of PEG-IFN- α conjugates are obtained (see also Monkarsh et al., ACS Symp. Ser., 680:207-216, 1997).

Kinstler et al. in European Patent Application EP 675 201 demonstrated the selective modification of the N-terminal residue of megakaryocyte growth and development factor (MGDF) with mPEG-propionaldehyde. This allowed for reproducible PEGylation and pharmacokinetics from lot to lot. Gilbert et al. in U.S. Patent 5,711,944 demonstrated that PEGylation of IFN- α with an optimal level of activity could be produced. In this instance a laborious purification step was needed to obtain the optimal conjugate.

The majority of cytokines, as well as other proteins, do not possess a specific PEG attachment site and, apart from the examples mentioned above, it is very likely that some of the isomers produced through the PEGylation reaction be partially or

totally inactive, thus causing a loss of activity of the final mixture.

Site-specific mono-PEGylation is thus a desirable goal in the preparation of such protein conjugates.

5 Woghiren et al. in Bioconjugate Chem., 4(5):314-318, 1993, synthesized a thiol-selective PEG derivative for such a site-specific PEGylation. A stable thiol-protected PEG derivative in the form of an orthopyridyl disulfide reactive group was shown to specifically conjugate to the free cysteine
10 in the protein, papain. The newly formed disulfide bond between papain and PEG could be cleaved under mild reducing conditions to regenerate the native protein.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or
15 considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicants at the time of filing and does not constitute an admission as to the correctness of such a statement.

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SUMMARY OF THE INVENTION

In the present invention, polyol-IFN- β conjugates, and particularly PEG-IFN- β conjugates, are provided wherein a polyol unit is covalently bound to Cys¹⁷. The specific conjugation is obtained by allowing a thiol-reactive polyol agent to react with
25 the Cys¹⁷ residue in IFN- β . Such conjugates are expected to show increased effectiveness *in vivo*. The aim is to obtain increased solubility at neutral pH, increased stability (decreased aggregation), decreased immunogenicity, and no loss of activity with respect to 'native' IFN- β . The results of such conjugation
30 would decrease the number of doses for an intended effect, simplify and stabilize the formulation of a pharmaceutical composition, and possibly increase the long-term efficacy.

The present invention further provides a method for the stepwise attachment of PEG moieties in series to a
35 polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the Capillary Electrophoresis (CE) graph of the PEG-IFN- β conjugate prior to purification.

Figures 2A-2C show the purification of the PEG-IFN- β conjugate carried out by size exclusion chromatography (Superose 12): Fig. 2A - first pass; Fig. 2B - second pass; Fig. 2C - third pass.

Figure 3 shows the SDS-PAGE chromatography of purified PEG-IFN- β conjugate from the third pass of chromatography. Lanes 1 and 4 are protein molecular weight standards, lane 2 is "native" IFN- β , and lane 3 is PEG-IFN- β conjugate.

Figure 4 reports the Capillary Electrophoresis (CE) graph of purified PEG-IFN- β conjugate in which IFN- β is PEGylated with mPEG-OPSS_{5k}.

Figure 5 reports the MALDI MS spectrum of purified PEG-IFN- β conjugate.

Figure 6 shows a comparison between the anti-viral activity of "native" IFN- β and of PEG-IFN- β conjugate. WISH cells were incubated with indicated concentrations of IFN- β samples for 24 hours prior to challenge with cytopathic dose of vesicular stomatitis virus. The cytopathic effect was determined after an additional 48 hours by MTT conversion.

Figure 7 shows the binding profile of IFN- β and PEG-IFN in Daudi cells.

Figure 8 shows the pharmacokinetic profile of IFN- β and PEG-IFN in mice following intravenous administration. The dotted lines indicate assay LOQ for each standard curve.

Figure 9 shows the pharmacokinetic profile of IFN- β and PEG-IFN in mice following subcutaneous administration. The dotted lines indicate assay LOQ for each standard curve.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that the attachment of a polyol moiety, more specifically a PEG moiety, to the Cys¹⁷ residue of human IFN- β unexpectedly increased (or at least retained and did not result in a decrease) the IFN- β biological activity from that of native

human interferon- β . Thus, not only does IFN- β with a polyol moiety attached to the Cys¹⁷ residue exhibit the same or increased IFN- β biological activity but this polyol-IFN- β conjugate also provides the desirable properties conferred by the polyol moiety, such as increased solubility.

"IFN- β ", as used herein, means human fibroblast interferon, as obtained by isolation from biological fluids or as obtained by DNA recombinant techniques from prokaryotic or eukaryotic host cells as well as its salts, functional derivatives, precursors and active fractions, provided that they contain the cysteine residue appearing at position 17 in the naturally occurring form.

The polyol moiety in the polyol-IFN- β conjugate according to the present invention can be any water-soluble mono- or bifunctional poly(alkylene oxide) having a linear or branched chain. Typically, the polyol is a poly(alkylene glycol) such as poly(ethylene glycol) (PEG). However, those of skill in the art will recognize that other polyols, such as, for example poly(propylene glycol) and copolymers of polyethylene glycol and polypropylene glycol, can be suitably used.

As used herein, the term "PEG moiety" is intended to include, but is not limited to, linear and branched PEG, methoxy PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, dendrimer PEG, copolymers of PEG and one or more polyols, and copolymers of PEG and PLGA (poly(lactic/glycolic acid)).

The definition "salts" as used herein refers both to salts of the carboxyl-groups and to the salts of the amino functions of the compound obtainable through known methods. The salts of the carboxyl-groups include inorganic salts as, for example, sodium, potassium, calcium salts and salts with organic bases as those formed with an amine as triethanolamine, arginine or lysine. The salts of the amino groups included for example, salts with inorganic acids as hydrochloric acid and with organic acids as acetic acid.

The definition "functional derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the terminal N- or C- groups according to known methods

and are included in the present invention when they are pharmaceutically acceptable, i.e., when they do not destroy the protein activity or do not impart toxicity to the pharmaceutical compositions containing them. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alkanoyl- or aroyl-groups.

The "precursors" are compounds which are converted into IFN- β in the human or animal body.

As "active fractions" of the protein, the present invention refers to any fragment or precursor of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example, residues of sugars or phosphates, or aggregates of the polypeptide molecule when such fragments or precursors show the same activity of IFN- β as medicament.

The conjugates of the present invention can be prepared by any of the methods known in the art. According to an embodiment of the invention, IFN- β is reacted with the PEGylating agent in a suitable solvent and the desired conjugate is isolated and purified, for example, by applying one or more chromatographic methods.

"Chromatographic method" means any technique that is used to separate the components of a mixture by their application on a support (stationary phase) through which a solvent (mobile phase) flows. The separation principles of the chromatography are based on the different physical nature of stationary and mobile phase.

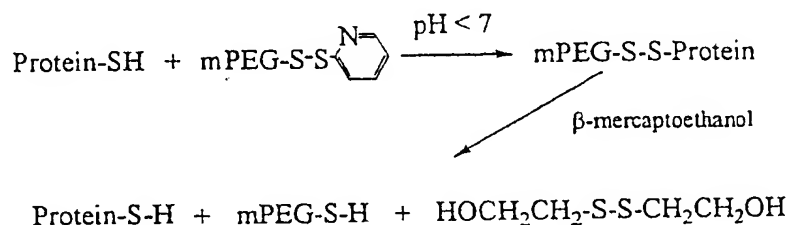
Some particular types of chromatographic methods, which are well-known in the literature, include: liquid, high pressure liquid, ion exchange, absorption, affinity, partition, hydrophobic, reversed phase, gel filtration, ultrafiltration or thin-layer chromatography.

The "thiol-reactive PEGylating agent", as used in the present application, means any PEG derivative which is capable of reacting with the thiol group of the cysteine residue. It can be, for example, PEG containing a functional group such as

orthopyridyl disulfide, vinylsulfone, maleimide, iodoacetimide, and others. According to a preferred embodiment of the present invention, the thiol-reactive PEGylating agent is the orthopyridyl disulfide (OPSS) derivative of PEG.

5 The PEGylating agent is used in its mono-methoxylated form where only one terminus is available for conjugation, or in a bifunctional form where both termini are available for conjugation, such as for example in forming a conjugate with two IFN- β covalently attached to a single PEG moiety. It has
10 preferably a molecular weight between 500 and 100,000.

A typical reaction scheme for the preparation of the conjugates of the invention is presented below:



The second line of the above scheme reports a method
15 for cleaving the PEG-protein linkage. The mPEG-OPSS derivative is highly selective for free sulphydryl groups and reacts rapidly under acidic pH conditions where the IFN- β is stable. The high selectivity can be demonstrated from the reduction of the conjugate to the native form of IFN- β and PEG.

20 The disulfide bond that is produced between the protein and PEG moieties has been shown to be stable in the circulation, but it can be reduced upon entering the cell environment. Therefore it is expected that this conjugate, which does not enter the cell, will be stable in the circulation
25 until it is cleared.

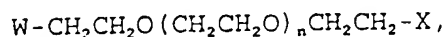
It should be noted that the above reaction is site-specific because the other two Cys residues appearing at positions 31 and 141 in the naturally occurring form of human IFN- β do not react with the thiol-reactive PEGylating agent
30 since they form a disulfide bridge.

The present invention is also directed to a method for the stepwise attachment of two or more PEG moieties to a polypeptide. This method is based upon the recognition that a low molecular weight activated PEG reacts more completely with a sterically hindered reaction site on a protein than does a high molecular weight activated PEG. PEG-modification of expensive therapeutic proteins must be cost effective in order for the production of the PEG conjugate to be practical. In addition, in order to reduce glomerular filtration and optimize the pharmacological properties of the PEG-protein conjugate, the conjugate should have an effective size equivalent to that of a protein with a molecular weight of 70 kDa. This means that for a site specific modification where one PEG will be attached, a PEG derivative having a molecular weight of greater than 20 kDa is preferably attached. If the site of modification is sterically crowded, the reactive group on the large PEG moiety may have difficulty reaching the modification site and thus will lead to low yields. A preferred method of PEGylating a polypeptide according to the present invention increases the yield of site-specific PEGylation by first attaching a small hetero or homobifunctional PEG moiety that, due to its relatively smaller size, can react with sterically crowded sites. Subsequent attachment of a large molecular weight PEG derivative to the small PEG results in high yield of the desired PEGylated protein.

The method for stepwise attachment of two or more PEG moieties in series to a polypeptide according to the present invention includes attaching a low molecular weight heterobifunctional or homobifunctional PEG moiety first to the polypeptide and then attaching a monofunctional or bifunctional PEG moiety to the free terminus of the low molecular weight PEG moiety that is attached to the polypeptide. Following the stepwise attachment of two or more PEG moieties in series to a polypeptide, which polypeptide is preferably IFN- β and where Cys¹⁷, located in a sterically crowded site, is the preferred site of PEG attachment, the PEG-polypeptide conjugate can be purified using one or more of the purification techniques such as ion exchange chromatography, size exclusion chromatography,

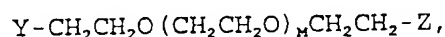
hydrophobic interaction chromatography, affinity chromatography, and reverse phase chromatography.

The low molecular weight PEG moiety has the formula:



5 where W and X are groups that independently react with an amine, sulfhydryl, carboxyl or hydroxyl functional group to attach the low molecular weight PEG moiety to the polypeptide. W and X are preferably independently selected from orthopyridyl disulfide, maleimides, vinyl sulfones, iodoacetamides, amines, thiols,
10 carboxyls, active esters, benzotriazole carbonates, p-nitrophenol carbonates, isocyanates, and biotin. The low molecular weight PEG moiety preferably has a molecular weight in the range of about 100 to 5,000 daltons.

The monofunctional or bifunctional PEG moiety for
15 attachment to the free terminus of a low molecular weight PEG that is attached to the polypeptide has preferably a molecular weight in the range of about 100 daltons to 200 kDa and is preferably a methoxy PEG, branched PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, or dendrimer PEG.
20 The monofunctional or bifunctional PEG furthermore has the formula:



where Y is reactive to a terminal group on the free terminus of the low molecular weight PEG moiety that is attached to the
25 polypeptide and Z is $-OCH_3$, or a group reactive with to form a bifunctional conjugate.

The PEG-polypeptide conjugate produced by the above method for stepwise attachment of two or more PEG moieties can be used to produce a medicament or pharmaceutical composition
30 for treating diseases or disorders for which the polypeptides is effective as an active ingredient.

Another object of the present invention is to provide the conjugates in substantially purified form in order for them to be suitable for use in pharmaceutical compositions, as active

ingredient for the treatment, diagnosis or prognosis of bacterial and viral infections as well as autoimmune, inflammatory diseases and tumors. Such pharmaceutical compositions represent a further object of the present invention.

Non-limiting examples of the above-mentioned diseases include: septic shock, AIDS, rheumatoid arthritis, lupus erythematosus and multiple sclerosis.

Further embodiments and advantages of the invention will be evident in the following description.

An embodiment of the invention is the administration of a pharmacologically active amount of the conjugates of the invention to subjects at risk of developing one of the diseases reported above or to subjects already showing such pathologies.

Any route of administration compatible with the active principle can be used. Parenteral administration, such as subcutaneous, intramuscular or intravenous injection is preferred. The dose of the active ingredient to be administered depends on the basis of the medical prescriptions according to age, weight and the individual response of the patient.

The dosage can be between 10 μ g and 1 mg daily for an average body weight of 75 kg, and the preferable daily dose is between 20 μ g and 200 μ g.

The pharmaceutical composition for parenteral administration can be prepared in an injectable form comprising the active principle and a suitable vehicle. Vehicles for the parenteral administration are well known in the art and include, for example, water, saline solution, Ringer solution and/or dextrose. The vehicle can contain small amounts of excipients in order to maintain the stability and isotonicity of the pharmaceutical preparation. The preparation of the solutions can be carried out according to the ordinary modalities.

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

EXAMPLE 1: Preparation of PEG-IFN- β Conjugate

5 Modification of IFN- β with mPEG_{5K}-OPSS

Recombinant human IFN- β , stable at a concentration of 0.37 mg/ml in 50 mM sodium acetate buffer, pH 3.6, was used for the preparation of a PEG-IFN- β conjugate. Approximately 1.0 ml of 6 M urea was added to 2 ml of IFN- β at a concentration of 0.37 mg/ml (0.74 mg, 3.7×10^{-8} moles). mPEG_{5K}-OPSS was added in a molar excess of 50 moles to one mole of IFN- β and the two were allowed to react in a polypropylene vial for either 2 hours at 37°C or 1 hour at 50°C. The reaction mixture was analyzed with Capillary Electrophoresis (CE) graph to determine the extent of PEG-IFN- β conjugate formation by the PEGylation reaction prior to any purification (Fig. 1). A typical yield for this reaction is 50% PEG-IFN- β . The reaction products were filtered from the reaction mixture with a 0.22 μ m syringe filter and the filtered solution was then loaded onto a size exclusion column (either Superose 12 or Superdex 75, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. Fig. 2A shows the elution profile from the purification of the PEG-IFN- β conjugate on a Superose 12 size exclusion chromatography column. The peaks were collected and analyzed with SDS-PAGE (Fig. 3). The fractions containing the PEG-IFN- β conjugate were pooled together and concentrate was then reloaded to the same size exclusion column to further purify the PEG-IFN- β conjugate due to the close proximity of the "native" IFN- β peak (Fig. 2B). This procedure was repeated (third pass) to ensure purity (Fig. 2C). Fig. 4 and Fig. 5 show the Capillary Electrophoresis graph and the MALDI MS spectrum, respectively, of the purified PEG-IFN- β conjugate.

Modification of IFN- β with mPEG_{30K}-OPSS

Recombinant human IFN- β was provided is stable in solution at 0.36 mg/ml in 50 mM sodium acetate buffer, pH 3.6.

Approximately 36 mg of mPEG_{30K}-OPSS in 3 ml deionized H₂O was added to 3 ml of IFN- β at 0.36 mg/ml (1.08 mg, 4.9×10^{-8} moles) and the two were allowed to react in a polypropylene vial for 2 hours at 50°C. The reaction mixture was analyzed with capillary electrophoresis for extent of modification. Typical yields for this reaction are <30%. The solution was then loaded onto a size exclusion column (Superose 12, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE for their contents.

10 EXAMPLE 2: Biological Activity of the PEG-IFN- β Conjugate

To assess the effects of PEGylation on the anti-viral activity of human recombinant IFN- β , human WISH amniotic cells were preincubated with either freshly prepared IFN- β (same lot as used for PEGylation) or PEG-IFN- β conjugate. The IFN- β -mediated anti-viral activity, as measured by the WISH-VSV cytopathicity assay, was determined according to an anti-viral WISH bioassay developed based on the protocol of Novick et al., J. Immunol., 129:2244-2247 (1982). The materials used in this WISH assay is as follows:

- 20 WISH cells (ATCC CCL 25)
Vesicular Stomatitis Virus stocks (ATCC V-520-001-522), stored at -70°C
IFN- β , human recombinant, InterPharm Laboratories LTD (32,075-type, Batch # 205035), 82×10^6 IU/ml, specific activity: 222×10^6 IU/mg
25 PEG-IFN- β conjugate as prepared in Example 1 and maintained in PBS, pH 7.4
WISH Growth medium (MEM high glucose with Earls salts + 10% FBS + 1.0% L-glutamine + Penicillin/Streptomycin (100 U/ml, 100 μ g/ml)
30 WISH Assay medium (MEM high glucose with Earls salts + 5% FBS + 1.0% L-glutamine + Penicillin/Streptomycin (100 U/ml, 100 μ g/ml)
MTT at 5 mg/ml in PBS, stored at minus 70°C.
35 The protocol for the WISH assay is as follows:
Dilute the IFN- β samples to 2X the starting concentration in WISH assay medium.

Make three-fold dilutions of IFN- β samples in WISH assay medium in flat-bottomed 96-well plate so that each well contains 50 μ l of diluted IFN- β sample (some control wells receive 50 μ l of WISH assay medium only).

- 5 Harvest log growth phase WISH cells with trypsin/EDTA solution, wash in WISH assay medium, and bring to a final concentration of 0.8×10^6 cells/ml.

Add 50 μ l of WISH cell suspension (4×10^4 cells per well) to each well. Final concentration of IFN- β exposed to the 10 cells is now 1X.

After incubation for 24 hours in a 5% CO₂ humidified incubator, 50 μ l of a 1:10 dilution (in WISH assay medium) of VSV stock (a dose predetermined to lyse 100 percent of WISH cells within 48 hours) is added to all wells except for the no 15 virus control wells (these receive an equal volume of assay medium only).

After an additional 48 hours, 25 μ l of MTT solution is added to all wells, after which plates are incubated for an additional 2 hours in an incubator.

- 20 The contents of the wells are removed by plate inversion, and 200 μ l of 100% ethanol is added to the wells.

After 1 hour, the plates are read at 595 nm using the Soft max Pro software package and Spectramax spectrophotometer system (Molecular Devices).

25 Table 1. Antiviral Activity of PEGylated and Mock-PEGylated IFN-beta Samples

IFN-beta Sample*	EC ₅₀ **
PEG-IFN- β conjugate	3.9 +/- 0.7 pg/ml
IFN- β	16.4 +/- 1.0 pg/ml

- 30 * Stock concentrations of IFN- β in samples determined by amino acid analysis.

** EC₅₀ (+/- S.D.) was determined by software program Microcal Origin 4.1

As demonstrated in Fig. 6 and Table 1 above, the PEG- 35 IFN- β conjugate maintained a level of anti-viral activity superior to that of the freshly prepared parental lot of IFN- β .

The observation that the PEG-IFN- β conjugate has approximately 4-fold higher bioactivity than that of freshly prepared IFN- β may be also a consequence of the increased stability of the PEG-IFN- β conjugate with respect to the "native" IFN- β after addition of WISH cell assay medium.

EXAMPLE 3: In Vitro Assays of the Relative Activity of PEG-IFN samples

Relative bioactivity of PEG[30 kD]-IFN- β and PEG[2 X 20 kD]-IFN- β was determined by WISH assay using the standard protocol described in Example 2 (Table 2). Three independent assays were performed by three different individuals at separate times.

Table 2. Relative antiviral activity of PEG-IFN- β

Sample	Relative Interferon Activity* (from three studies)			
	Assay 1	Assay 2	Assay 3	Average (S.D.)
PEG[30kD]-IFN- β	3.2 X higher	3.1 X higher	1.8 X higher	3.0 X (0.78) higher
PEG[2 x 20kD]-IFN- β	4.2 X higher	1.3 X higher	0.85 X higher	2.1 X (1.8) higher

*EC50 doses compared with standard IFN- β included in each assay.

**Comparison based on IFN- β concentration of 330 ug/ml. Stock concentrations of PEG[30 kD]-IFN- β (5.41 ug/ml) and PEG[2 x 20 kD]-IFN- β (6.86 ug/ml) were determined by AAA.

The binding of PEG-IFN- β to its receptor on cells was evaluated in the presence of a fixed amount of 125 I-IFN- α 2a. IFN- α 2a was radiolabeled with 125 I using the chloramine T method. The 125 I bound IFN α 2a was removed from free iodine by running the reactants through a Sephadex G25 column and pooling the protein containing fractions (Pharmacia). 125 I-IFN- α 2a was quantified by an IFN- α 2a ELISA assay (Biosource, USA) and the specific activity was determined. Daudi cells grown in the exponential phase of growth were harvested and 2×10^6 cells were incubated with 0.5 nM 125 I-IFN- α 2a for 3 hours at room temperature in the presence of different concentrations of PEG-IFN- β or IFN- α 2a diluted in an assay buffer which is RPMI 1640 containing 2%

fetal bovine serum and 0.1% sodium azide. At the end of the incubation, the cells were spun through a layer of phthalate oil and the cell bound radioactivity was counted on the gamma counter. Furthermore, the binding of PEG[30kD]-IFN- β and PEG[25 x 20kD]-IFN- β to the receptor were very similar or close to the binding activity of IFN- β as shown in Fig. 7.

In addition, relative activity was determined in a Daudi cell (human B cell lymphoma) anti-proliferation assay (Table 3). All IFNs were made at a 2x concentration of 200 ng/ml. Samples were diluted three-fold down the length of the plate at a final volume of 100 μ l. 1×10^5 cells/well (100 μ l) were added to each well and incubated for a total of 72 hours at 37°C in CO₂ humidified incubator. After 48 hours, tritiated (³H) thymidine were added at 1 μ Ci/well in 20 μ l. At the end of the 72 hour incubation, the plate was harvested with the Tomtek Plate Harvester. The results shown in Table 3 indicate that no detectable loss of IFN activity was observed from PEGylation. In fact, the activity was found to be somewhat higher than free IFN- β . This may be due to the formation of inactive aggregates in the free IFN or to the differences in quantitation methods (amino acid analysis for PEG-IFN samples and RP-HPLC for IFN- β).

Table 3. Daudi Anti-Proliferation Assay

	IC ₅₀ dose*	Fold increase vs IFN
IFN- β (Plate 1)	1153.1	—
PEG[30kD]-IFN (71 A)	695.6	1.6X
IFN- β (Plate 2)	1005.8	--
PEG[40kD]-IFN (71 B)	629.4	1.7X

*pg/ml

EXAMPLE 4: Pharmacokinetic Studies in Mice

Intravenous Administration

Mice were injected with 100 ng of IFN- β , PEG[30kD]-IFN- β or PEG[2 X 20 kD]-IFN- β and blood was removed at indicated times thereafter. Serum concentrations of IFN- β were determined

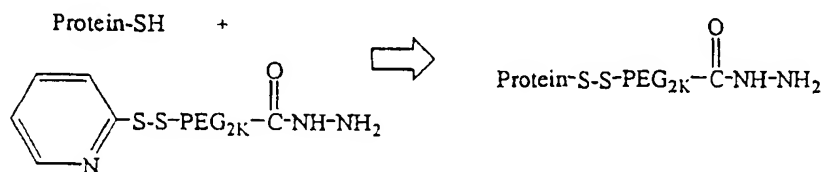
by IFN- β -specific ELIAS (Toray Industries) and the results are shown in Fig. 8. Twenty-eight female B6D2F1 strain mice (6-8 wks) (approximately 20g each) were separated into four groups as follows: Group 1 contained nine mice injected with a 200 μ l single bolus of 500 ng/ml human IFN- β (final dose of 100 ng/mouse); Group 2 (nine mice) received 200 μ l of an equivalent mass of PEG30kD-IFN- β ; Group 3 received 200 μ l of an equivalent mass of PEG(2 x 20 kD)-IFN- β ; and Group 4 is a group of three uninjected mice serving as a negative control. Blood samples (approximately 200 μ l/sample) were collected at nine indicated times by disruption of the retro-orbital venous plexus with a capillary tube. Blood samples were allowed to clot for 1 hr at room temperature, rimmed and microcentrifuged. Sera removed therefrom were stored at -70°C until all samples were collected. Sera were assayed for the presence of bioactive human IFN- β using the Toray assay. The results indicate that the area under the curve (AUC) is markedly enhanced in the PEG-IFN samples versus free IFN-beta and that PEG-IFN samples versus free IFN- β and that PEG[2 X 20 kD]-IFN- β is superior to the PEG[30 kD]-IFN- β .

Subcutaneous Administration

Mice were injected subcutaneously with IFN- β and PEG-IFN (100 ng/mouse). Figure 9 demonstrates that the total area under the curve (AUC) is dramatically enhanced for the PEG-IFN samples as compared with free IFN- β . The pharmacokinetic studies are consistent with the PEG-IFN samples having a longer half-life and increased AUC.

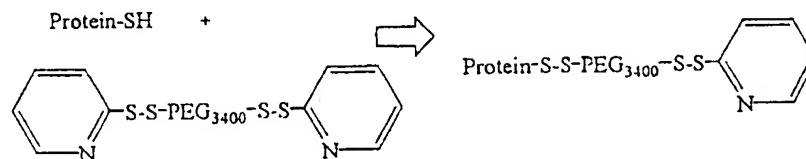
Example 5: Attachment of Low Molecular Weight PEG Moiety to Polypeptide

30 Tagging Interferon-beta with OPSS-PEG_{2K}-Hydrazide



Recombinant human interferon- β was provided in solution at 0.33 mg/ml in 50mM sodium acetate buffer, pH 3.8. Approximately 3.6 mg (40 mole excess to moles of protein) of the heterobifunctional PEG reagent, OPSS-PEG_{2k}-hydrazide, in 2 ml deionized water was added to 3 ml of IFN- β at 0.33 mg/ml (0.99 mg) and the two were allowed to react in a polypropylene vial for 1 hour at 45°C. The reaction mixture was then analyzed with capillary electrophoresis to determine the extent of modification. Typical yields ranged from 90-97% that depended on the purity of the interferon β and PEG reagent. The solution was next loaded onto a size exclusion column (Superdex 75, Pharmacia) and eluted with 5 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE. The monoPEGylated interferon- β fractions were pooled together than used in a further modification step with high molecular weight PEG.

Tagging Interferon- β with (OPSS)₂-PEG₃₄₀₀

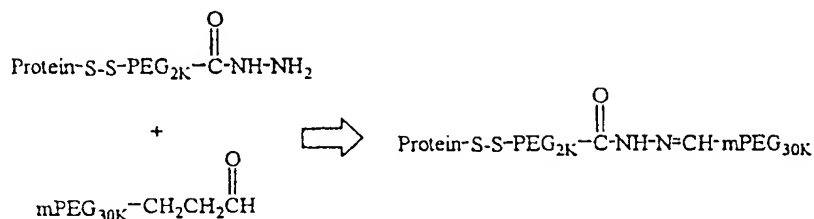


Recombinant human interferon- β was provided in solution at 0.33 mg/ml in 50 mM sodium acetate buffer, pH 3.8. Approximately 6.1 mg (40 mole excess to moles of protein) of the homobifunctional PEG reagent, (OPSS)₂-PEG₃₄₀₀, in 2 ml deionized water was added to 3 ml of interferon- β at 0.33 mg/ml (0.99 mg) and the two were allowed to react in a polypropylene vial for 2 hours at 50°C. The reaction was monitored with non-reducing SDS-PAGE and the final reaction mixture was analyzed with capillary electrophoresis to determine the extent of modification. Typical modifications for this reaction with interferon- β were >95%. The solution was then loaded onto a

size exclusion column (Superdex 75, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE for their contents. The monoPEGylated interferon- β fractions were combined.

5 EXAMPLE 6: Attachment of Second PEG Moiety to Low Molecular Weight PEGylating Polypeptide

Modification of IFN-S-S-PEG_{2k}-Hydrazide with mPEG_{30k}-Aldehyde (ALD)



To the combined fractions of IFN-S-S-PEG_{2k}-Hydrazide in
 10 Example 5 was added mPEG_{30k}-ALD in a 20 mole excess to protein.
 The reaction was conducted at room temperature (25°C) for 4
 hours and a sample was added to a size exclusion column
 (Superose 6, Pharmacia) to determine modification yield. The
 modification yield of this reaction was typically >80% depending
 15 upon the purity of the PEG reagent and reaction conditions.

Having now fully described this invention, it will be
 appreciated that by those skilled in the art that the same can
 be performed within a wide range of equivalent parameters,
 concentrations, and conditions without departing from the spirit
 20 and scope of the invention and without undue experimentation.

While this invention has been described in connection
 with specific embodiments thereof, it will be understood that it
 is capable of further modifications. This application is
 intended to cover any variations, uses, or adaptations of the
 25 inventions following, in general, the principles of the
 invention and including such departures from the present
 disclosure as come within known or customary practice within the
 art to which the invention pertains and as may be applied to the

essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign
5 patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references.. Additionally, the entire contents of the references cited within the references cited herein are also
10 entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the
15 relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily
20 modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments,
25 based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and
30 guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

WHAT IS CLAIMED IS:

1. A polyol-interferon- β conjugate having a polyol moiety covalently bound to Cys¹⁷ of human interferon- β .

2. The polyol-interferon- β conjugate according to claim 1, wherein said polyol moiety is a polyalkylene glycol moiety.

3. The polyol-interferon- β conjugate according to claim 2, wherein said polyalkylene glycol moiety is a polyethylene glycol (PEG) moiety.

4. The polyol-interferon- β conjugate according to any of claims 1-3, wherein the polyol-interferon- β conjugate has the same or higher interferon- β activity as native human interferon- β .

5. A process for producing the polyol-interferon- β conjugate of claim 1, comprising the steps of:

reacting interferon- β with a thiol-reactive polyol agent to site specifically and covalently attach a polyol moiety to Cys¹⁷ of human interferon- β to produce a polyol-interferon- β conjugate; and

recovering the produced polyol-interferon- β conjugate.

6. The process according to claim 5, wherein the thiol-reactive polyol agent is a thiol-reactive PEGylating agent.

7. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is mono-methoxylated.

8. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is bifunctional.

9. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is a polyol derivative having a functional group selected from the group consisting of orthopyridyl disulfide, vinyl sulfone, maleimide, and iodoacetimide.

10. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is an orthopyridyl disulfide derivative of a mono-methoxylated polyol.

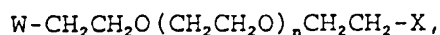
11. The process according to claim 5, wherein the reacting step is carried out at an acidic pH where interferon- β is stable.

12. A pharmaceutical composition, comprising a polyol-interferon- β conjugate according to any one of claims 1-3, as an active ingredient, and a pharmaceutically acceptable carrier, excipient or auxiliary agent.

13. A method for treating infections, tumors and autoimmune and inflammatory diseases, comprising administering an effective amount of the pharmaceutical composition according to claim 12 to a subject in need thereof.

14. A method for stepwise attachment of polyethylene glycol (PEG) moieties in series to a polypeptide, comprising the steps of:

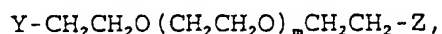
reacting a polypeptide with a low molecular weight heterobifunctional or homobifunctional PEG moiety having the following formula:



where W and X are groups that independently react with an amine, sulfhydryl, carboxyl or hydroxyl functional group to attach the low molecular weight PEG moiety to the polypeptide; and

reacting the low molecular weight PEG moiety attached to the polypeptide with a monofunctional or bifunctional PEG moiety to attach the monofunctional or bifunctional PEG moiety to a free terminus of the low molecular weight PEG moiety and form a PEG-polypeptide conjugate.

15. The method according to claim 14, wherein the monofunctional or bifunctional PEG moiety has the following formula:



wherein Y is reactive to a terminal group on the free terminus of the low molecular weight PEG moiety attached to the polypeptide and Z is $-OCH_3$ or a group reactive with X to form a bifunctional conjugate.

16. The method according to claim 15, wherein the monofunctional or bifunctional PEG moiety is methoxy PEG, branched PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, or dendrimer PEG.

17. The method according to claim 14, wherein W and X are independently selected from the group consisting of orthopyridyl disulfide, maleimides, vinylsulfones, iodoacetamides, hydrazides, aldehydes, succinimidyl esters, epoxides, amines, thiols, carboxyls, active esters, benzotriazole carbonates, p-nitrophenol carbonates, isocyanates, and biotin.

18. The method according to claim 14, wherein the low molecular weight PEG moiety has a molecular weight in a range of about 100 to 5,000 daltons.

19. The method according to claim 14, wherein the monofunctional or bifunctional PEG moiety has a molecular weight in a range of about 100 daltons to 200 kilodaltons.

20. The method according to claim 14, wherein the low molecular weight PEG moiety and/or the monofunctional or bifunctional PEG moiety is a copolymer of polyethylene glycol.

21. The method according to claim 20, wherein the copolymer of polyethylene glycol is selected from the group consisting of polyethylene glycol/polypropylene glycol copolymers and polyethylene glycol/poly(lactic/glycolic acid) copolymers.

22. The method according to claim 14, further comprising a step of purifying the PEG-polypeptide conjugate following the stepwise attachment of two PEG moieties in series to a polypeptide.

23. The method according to claim 22, wherein said step of purifying comprises one or more purification techniques selected from the group consisting of ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, affinity chromatography, and reverse phase chromatography.

24. The method according to any one of claim 14-23, wherein the polypeptide is interferon- β .

25. Use of the PEG-polypeptide conjugates produced by the method according to any one of claims 14-23 as a medicament.

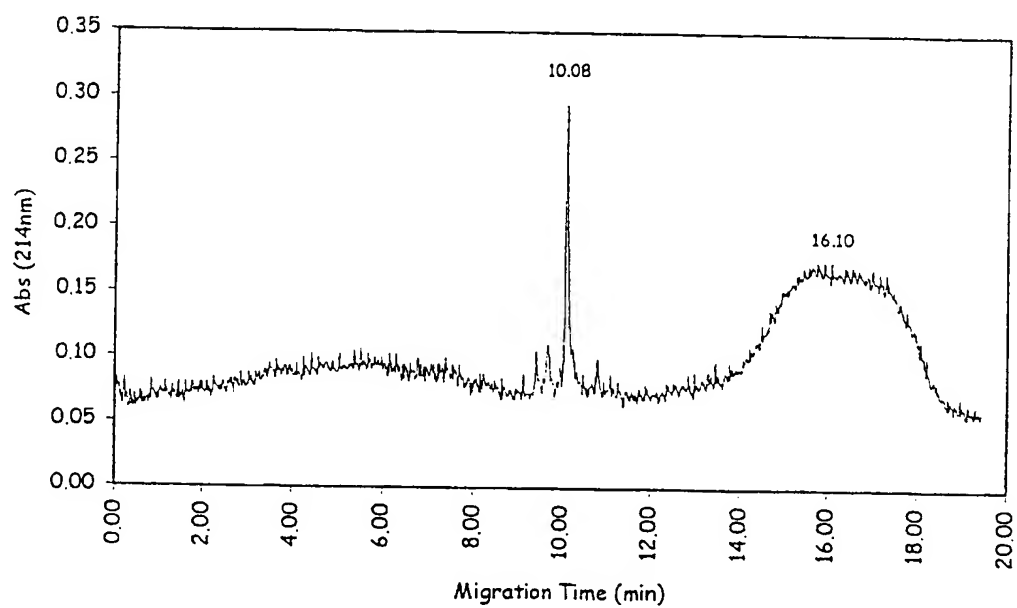


FIG. 1

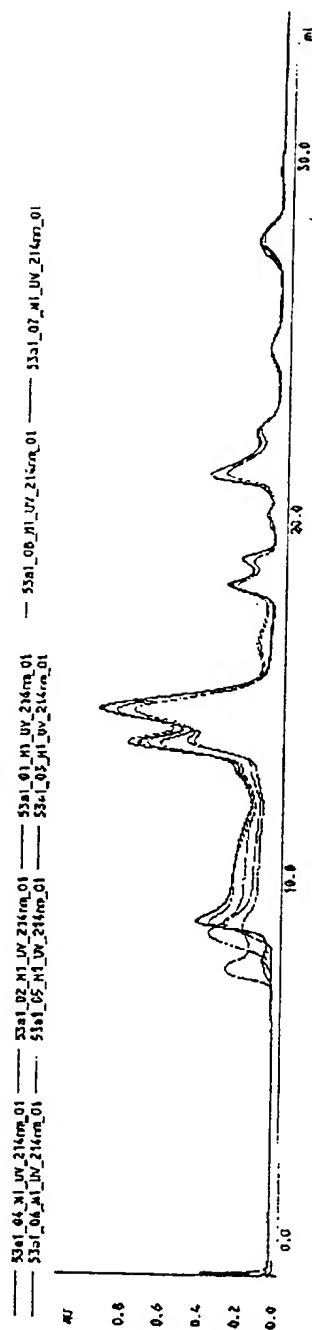


FIG. 2A

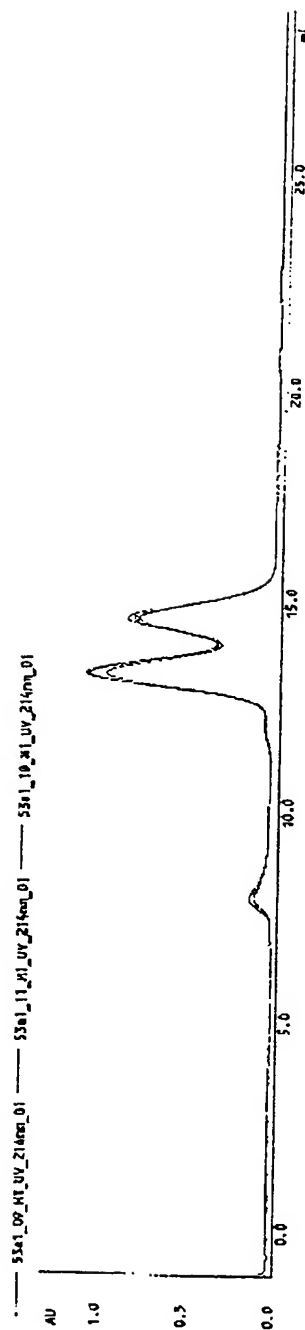


FIG. 2B

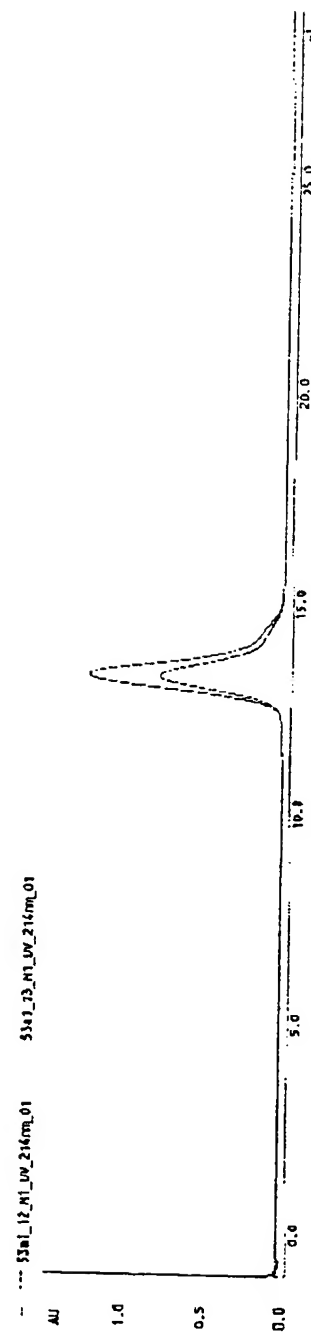


FIG. 2C

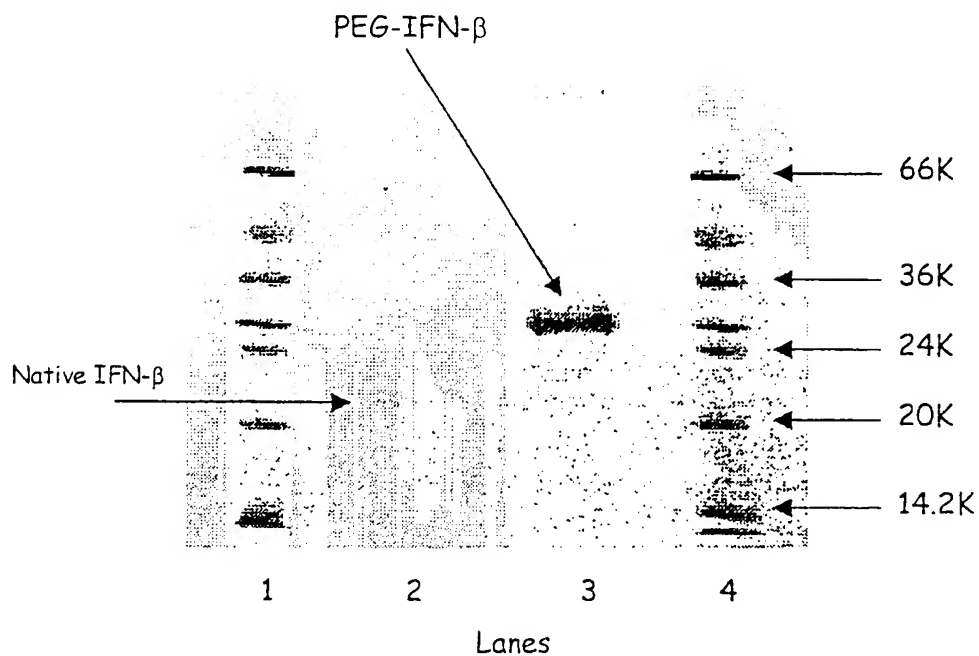


FIG. 3

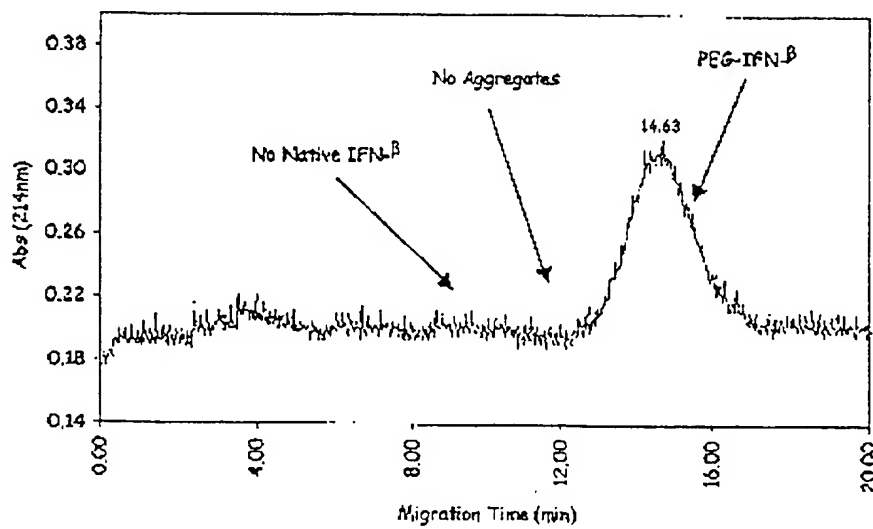


FIG. 4

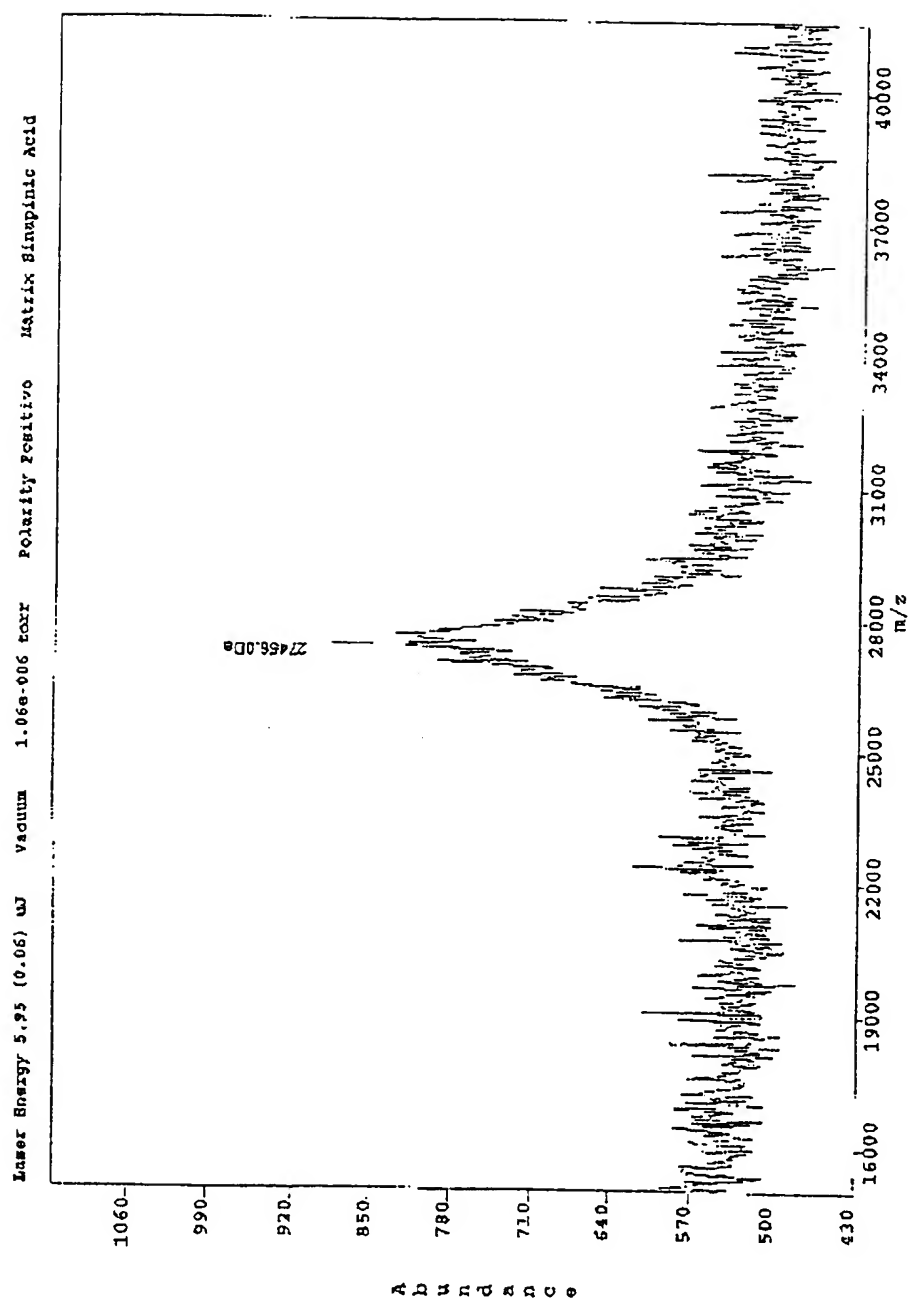


FIG. 5

O.D. at 595 nm

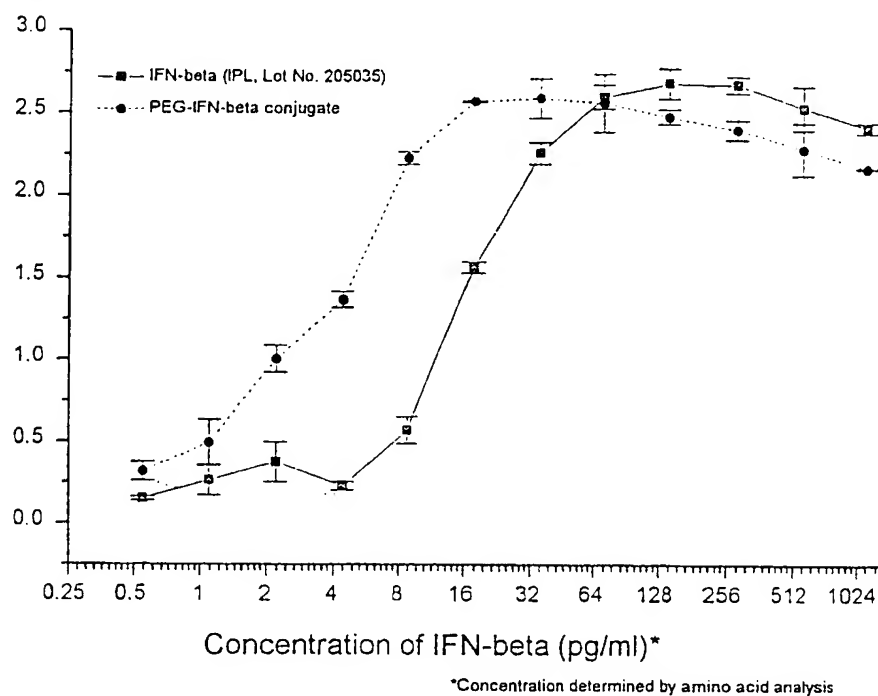


FIG. 6

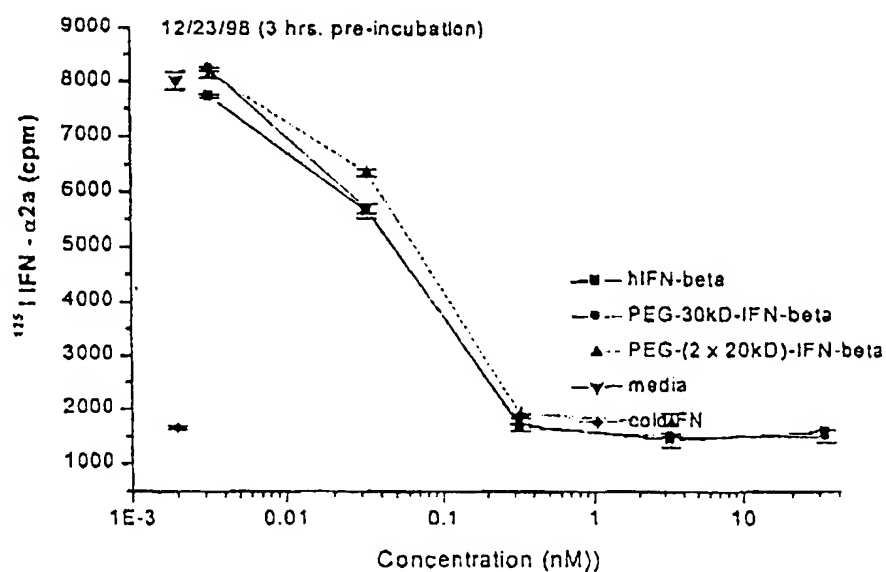


FIG. 7

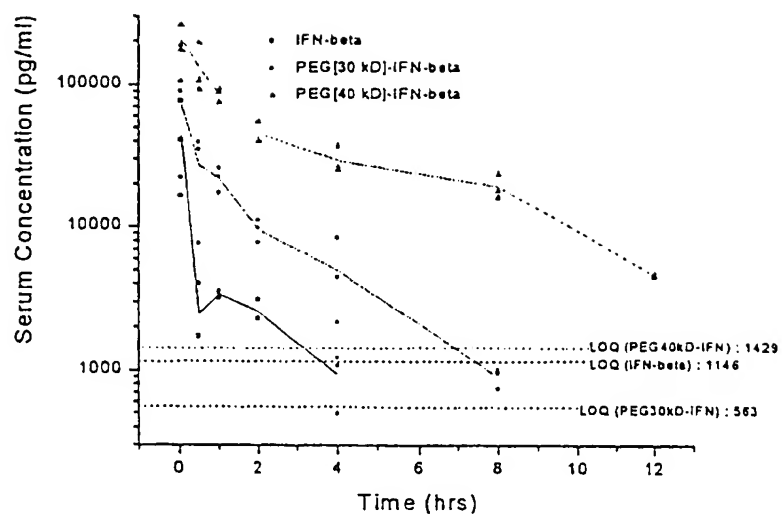


FIG. 8

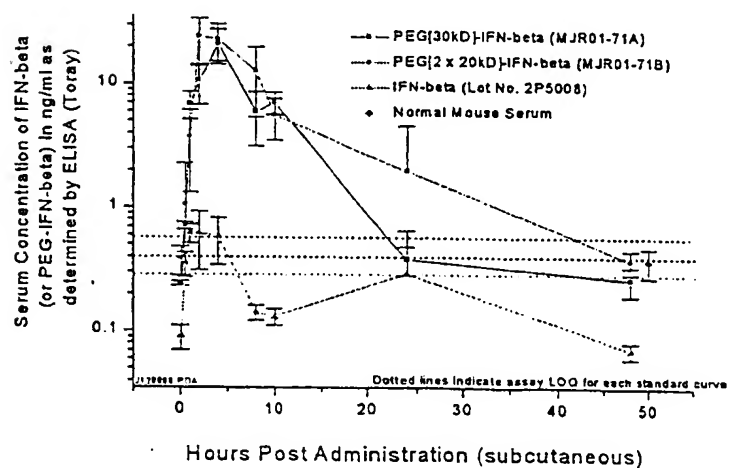


FIG. 9

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US99/09161</p> <p>(22) International Filing Date: 28 April 1999 (28.04.99)</p> <p>(30) Priority Data: 60/083,339 28 April 1998 (28.04.98) US</p> <p>(71) Applicant (for all designated States except US): APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. [NL/NL]; 14 John B. Gorsiraweg, Curacao (AN).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): EL TAYAR, Nabil [CH/US]; 143 Gerald Road, Milton, MA 02186 (US). ROBERTS, Michael, J. [US/US]; 104 Cane Brook Court, Madison, AL 35758 (US). HARRIS, Milton [US/US]; 3119 Highland Plaza, Huntsville, AL 35801 (US). SAWLIVICH, Wayne [US/US]; 24 Morse Avenue, Wilmington, MA 01887 (US).</p> <p>(74) Agent: YUN, Allen, C.; Browdy and Neimark, P.L.L.C., Suite 300, 419 Seventh Street, N.W., Washington, DC 20004 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p> <p>(88) Date of publication of the international search report: 29 December 1999 (29.12.99)</p>
<p>(54) Title: POLYOL-IFN-BETA CONJUGATES</p> <p>(57) Abstract</p> <p>PEG-IFN-β conjugates, where a PEG moiety is covalently bound to Cys¹⁷ of human IFN-β, are produced by a process of site specific PEGylation with a thiol reactive PEGylating agent. A pharmaceutical composition and a method for treating infections, tumors and autoimmune and inflammatory diseases are also provided. The invention further relates to a method for the stepwise attachment of PEG moieties in series to a polypeptide, and more particularly to IFN-β.</p>		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/09161

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOODSON R J ET AL: "SITE-DIRECTED PEGYLATION OF RECOMBINANT INTERLEUKIN-2 AT ITS GLYCOSYLATION SITE" BIO/TECHNOLOGY, vol. 8, no. 4, 1 April 1990 (1990-04-01), pages 343-346, XP000563746 ISSN: 0733-222X abstract	1-25
X		14,15, 17-19, 22-24
Y	WO 87 00056 A (CETUS CORP) 15 January 1987 (1987-01-15) page 30-32; examples VI-VII	1-13
	-/-	



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- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

13 October 1999

Date of mailing of the international search report

22. 10. 99

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Herrera, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09161

C. Continuation; DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	US 5 206 344 A (KATRE NANDINI ET AL) 27 April 1993 (1993-04-27) abstract ---	1-13
Y	US 5 166 322 A (SHAW GRAY ET AL) 24 November 1992 (1992-11-24) abstract ---	1-13
P,X	WO 99 03887 A (BOLDER BIOTECHNOLOGY INC ;COX GEORGE N III (US)) 28 January 1999 (1999-01-28) page 41, line 26 -page 42, line 18 page 4, line 31 -page 5, line 24 ---	1-13
P,X	WO 98 32466 A (FRANCIS GILLIAN ELIZABETH ;FISHER DEREK (GB); MALIK FAROOQ (GB); P) 30 July 1998 (1998-07-30) page 15, line 9 -page 16, line 19; claims 1,2,7 ---	14,15, 18,19, 24,25
Y	---	14-25
X	WO 97 11957 A (PHARMACIA & UPJOHN AB ;DALBORG JOHANNA (SE); SANDBERG HELENA (SE);) 3 April 1997 (1997-04-03) page 10, line 19 -page 11, line 18; claims 1-25 ---	14-25
Y	EP 0 690 127 A (AMGEN INC) 3 January 1996 (1996-01-03) claims -----	14-23

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 99/09161

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos. :
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos. :
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos. :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 99/09161

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-13

Conjugates comprising interferon beta, which has a polyol covalently bound to Cys17 and processes for preparing them

2. Claims: 14-25

Process for attaching PEG stepwise to polypeptides

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/09161

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Information on patent family members

International Application No

PCT/US 99/09161

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